Non-Symbiotic Nitrogen-Fixing Organisms in Queensland Soils

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SUMMARY

The occurrence of Azotobacter chroococcum *and* Clostridium *spp. has been determined in a number of cultivated and virgin Queensland soils, representing a wide range in soil characteristics.*

Comparisons have been made of the Beijerinck solution, soil plaque and agar plate methods for the detection of Azotobacter. *The Beijerinck solution method is an accurate and sensitive test for determining the presence of* Azotobacter *and technical criticisms of this method appear to be without foundation. The organism may be detected as frequently by the soil plaque as by the agar plate method.*

The H^+ *concentration of the soil is a major factor governing the occurrence of* Azotobacter *in Queensland soils. The percentage of positive determinations in soils with pH values below* 5.9 *was lower than in soils with pH values above* 5.9.

A lower percentage of positive determinations was obtained when the soil moisture was below 10% *but there was no significant variation in percentage of positive determinations for various soil moisture ranges above* 10%.

An effect of soil type on the occurrence of Azotobacter *was revealed, an increasing percentage of positive samples being recorded in the order sands, sandy loams, loams, clay loams.*

The black earth soils are rich in Azotobacter. *The organism may be present in grey-brown soils of western Queensland but is generally absent from the granitic sands, the weakly podsolized coastal soils and the red earths.*

There was no indication that Azotobacter *occurred more frequently in virgin than in cultivated soils.*

Anaerobic species of Clostridium *were found constantly in Queensland soils.*

INTRODUCTION

The only soil bacteria, apart from the symbiotic organisms of the genus *Rhizobium*, conclusively proved capable of fixing nitrogen are aerobic bacteria of the genus *Azotobacter* and anaerobic bacteria of the genus *Clostridium*. Since 1895 microbiological investigations directed towards finding recuperative agencies for replenishing losses of nitrogen from the soil have almost entirely been confined to studies of species of these three genera.

While the economic importance of species of *Rhizobium,* in association with legumes, has been long established, the importance of the role played by the non-symbiotic nitrogen-fixing organisms in the nitrogen economy of soils has not yet been definitely assessed. Considerable research, however, has been carried out, since the discovery of the aerobic nitrogen-fixing *Azotobacter chroococcum* by Beijerinck (1901) and of the anaerobic nitrogen-fixing *Clostridium pasteurianum* by Winogradsky (1895), to determine the distribution of these and related species and to examine the effects of environmental factors on their occurrence.

Anaerobic species of *Clostridium* are generally present in soils. The occurrence of species of *Azotobacter,* on the other hand, appears to be governed by certain environmental factors (Waksman, 1932; Russell, 1932; Jensen, 1940).

No record of the isolation of *Azotobacter* or *Clostridium* from Queensland soils appears in the literature. Quantitative determinations in South Australia, Victoria and New South Wales indicate a sporadic occurrence of *Azotobacter* in those States, while species of *Clostridium* have been shown to occur almost constantly in the soils of Victoria and New South Wales. Previous Australian work is reviewed hereunder.

Darnell-Smith (1912) isolated *Azotobacter* from three soils in New South Wales. Lewcock (1925) reported the qualitative isolation of *Azotobacter* from a number of soils and suggested a widespread distribution of this organism in South Australian soils. Penman and Rountree (1932) found *Azotobacter* present in a soil under wheat and the same soil kept bare in Rutherglen, Victoria. Beck (1935) made determinations on 33 South Australian soils, 10 of which contained *Azotobacter.* Swaby (1939) examined 80 Victorian soils and found *Azotobacter* present in 21; *Clostridium butyricum* was present in the majority of soils examined. Jensen (1940) found *Azotobacter* present in 27 of 85 New South Wales soils, 72 of which came from the wheat belt. Anaerobic nitrogen-fixing bacteria of the butyric acid bacilli group were almost always present in these soils. Subsequently, Jensen and Swaby (1940) made further observations on the distribution of *Azotobacter* in New South Wales soils and found the organism in 10 soils out of a total of 58.

In the course of the present investigations, 368 soil samples have been examined qualitatively and quantitatively for *Azotobacter chroococcum,* and 172 soils qualitatively for *Clostridium.* In a study of the distribution of these organisms a wide range of cultivated and virgin soils has been examined from the major soil types of south-eastern Queensland, together with samples from other parts of the State and from New Guinea.

METHODS

Soil samples were taken to a depth of $5\frac{1}{2}$ inches in the field and brought to the laboratory in sterilized canisters. Laboratory determinations were made within four days on the majority of samples. The examination of soils collected in the Brisbane district was made within 24 hours.

Qualitative Determinations of Azotobacter by the Beijerinck Solution Method.

By inoculating 5 gm. or 7.5 gm. of soil into 45 ml. of sterile Beijerinck solution (mannitol, 2.0%; K_2HPO_4, 0.02% in tap water; plus 0.5 gm. $CaCO_3$ per flask) in 250 ml. Erlenmeyer flasks. Flasks were incubated in duplicate for seven days at 30° C., when the surface was examined macroscopically for the presence of the *Azotobacter* pellicle, and microscopically after staining with Ziehl-Neelsen carbol fuchsin for *Azotobacter* cells.

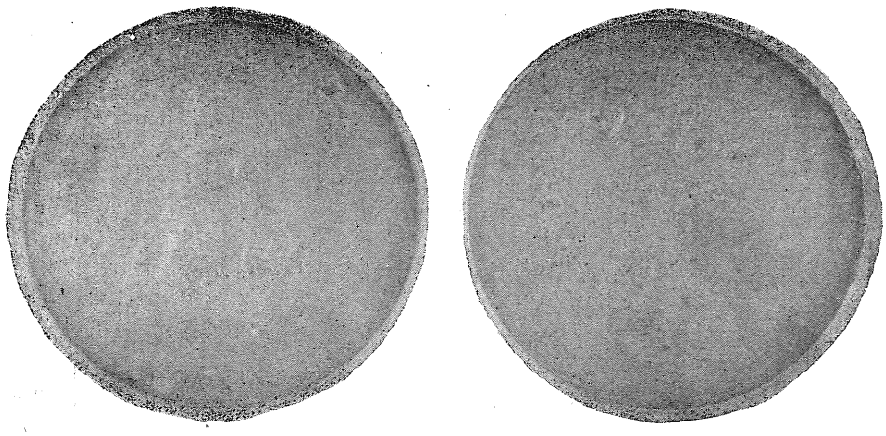

Figure 1

Azotobacter chroococcum. Soil plaques after 49 hours incubation. Left, positive; right, negative.

Qualitative Determinations of Azotobacter by the Winogradsky Soil Plaque Method.

Peterson and Goodding's (1941) modification of the Winogradsky soil plaque was employed. In this method 50.0 gm. of the soil to be tested are mixed with 2.5 gm. potato starch, 0.3 gm. K_2HPO_4, 0.2 gm. $CaCO_3$, 0.5 ml. of 0.5% ammonium nitrate, and sufficient molybdate solution containing 0.5 ml. of 1% ammonium molybdate per litre of water to make a well-moulded smooth-surfaced plaque in sterile 10 cm. petri dishes. The incubation period of 48 hours suggested by Peterson and Goodding was found insufficient with some soils, (Fig. 1), and final observations on plaques were made after incubation in duplicate in moist chambers for 96 hours at 30° C. In the case of sandy soils the duplicate plaque received the addition of 2.5 gm. sterile kaolin.

Quantitative Determinations of Azotobacter.

(a) By the plate count method devised by Swaby (1939) on the mannitol agar medium of Curie (1931). Formula: Mannitol, 20.0 gm.; agar, 18.0 gm.; and 2 gm. of the following stock mixture per litre of medium: K_2HPO_4, 100 parts; $MgSO_4$, 60 parts; NaCl, 60 parts; $Fe(SO_4)_3$, 1 part; $MnSO_4$. 1

part; $CaCO_3$, 178 parts. The procedure is to pour a layer of sterile agar in petri dishes, allow to cool, pipette 1 ml. of inoculum over the surface, and add 2 ml. of sterile mannitol agar at 42° C. This is mixed with the inoculum and the plates incubated in the inverted position.

(b) On dextrine agar (Fig. 2), using Jensen's (1940) technique. The formula for this agar is : Dextrine, 10.0 gm.; K_2HPO_4, 0.5 gm.; $MgSO_4$, 0.2 gm.; $FeCl_3$, 0.05 gm.; Na_2MoO_4, 0.025 gm.; $CaCO_3$, 5.0 gm.; agar, 20.0 gm.; H_2O, 1000 ml. The procedure is to pour a layer of sterile dextrine agar in 10 cm. petri dishes, allow to cool, and to pipette 0.4 ml. of soil-sterile water

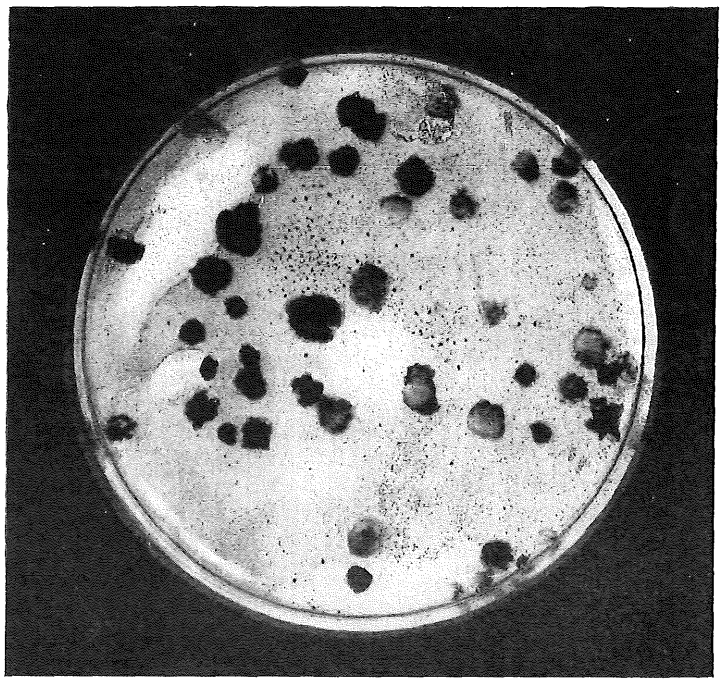

Figure 2
5-day-old dextrine agar plate of *Azotobacter chroococcum*.

suspension on to the surface. This is spread evenly over the surface with a stout L-shaped platinum wire. The plates are then allowed to stand with the tops removed until excess water has evaporated and are incubated in the inverted position.

The method of obtaining suspensions, standard throughout the determinations, was to shake the soil-sterile water suspensions in 250 ml. Erlenmeyer flasks in a horizontal cradle shaker, 120 shakes per minute, for 10 minutes. Soil-sterile water suspensions employed ranged from 1 : 10 to 1 : 200.

Counts were made on four parallel plates after five days' incubation at 30° C.

Qualitative Determinations of Clostridium.

By incubation in glucose solution (glucose, 10.0 gm.; K_2HPO_4, 0.5 gm.; $MgSO_4$, 0.2 gm.; NaCl, 0.2 gm.; $FeCl_3$, 0.02 gm.; $CaCO_3$, 10.0 gm.; and 1.0 gm. agar in 1000 ml. water ± trace of sodium molybdate or ammonium molybdate. Bacteriological test tubes were filled to a height of 10 cm. with this medium, sterilized and inoculated with soil suspension corresponding to 0.1 gm. of soil. Determinations of *Clostridium* were made, after incubation for a variable period at 25° C. or 30° C., by microscopic examination of the sediment after staining with Lugol's iodine solution. Spore determinations of *Clostridium* were made by heating inoculated tubes for 10 minutes at 85° C. before incubation.

pH Determinations:

By a saturated calomel-glass electrode system in conjunction with a Cambridge electrometer valve pH meter.

Moisture Determinations.

In duplicate by drying at 105° C. to constant weight.

Soil Classification.

Estimated from the texture as determined by the "feel" at optimum moisture capacity.

COMPARISON OF TECHNIQUES FOR DETERMINING THE PRESENCE OF AZOTOBACTER

The efficiency of cultural methods for the detection of *Azotobacter* has been variously reported by different workers.

Investigations on the occurrence of *Azotobacter* in soils have been made generally by the employment of one or other of the three cultural methods:—

(1) The Beijerinck solution method or one of its modifications. This method, while usually employed for qualitative determinations, may be used, employing a dilution method, to give approximate quantitative determinations.

(2) The Winogradsky soil plaque method or one of its modifications. While primarily a qualitative method, the soil plaque may be used to give approximate quantitative determinations by assessing the area of the plaque showing colonies and the intensity of growth.

(3) A silica-gel or, latterly, an agar plate method determining quantitatively the number of colonies of *Azotobacter* by inoculating the surface with a known weight of sieved soil, or the number of individual *Azotobacter* cells by using a soil-sterile water suspension as inoculum.

The direct microscopic method has been used in studies of *Azotobacter,* but apart from the inability to distinguish between dead and living cells and the probable existence of bacteria morphologically similar to *Azotobacter,* many

Queensland soils have *Azotobacter* populations of less than 10 cells per gram, rendering unreliable the use of the direct microscopic method for number determinations.

Considerable difference of opinion exists as to the efficacy of the solution method, and particularly of the number of cells required to produce a typical pellicle. Doubt as to the sensitivity of the solution method when a small number of cells is present has been expressed by Gainey (1923): "Little is known as to how many *Azotobacter* are necessary to initiate the development of a visible film in laboratory culture media. Unpublished data indicate that appreciable numbers are essential to the development of a characteristic film." On the other hand, Duggeli (1924) and Wenzl (1934), quoted by Jensen (1940), made counts of *Azotobacter* both on agar plates and by dilution in mannite solution and found that the solution method gave counts as high as, or even higher than, the plate method.

Vigorous criticism of the solution method has been made by Winogradsky (1928): "The method is certainly untrustworthy in negative cases, and it is quite incapable of yielding the slightest information concerning the density of *Azotobacter* cells and their state of activity in the natural soil. The method may retain its historical value but it is about time to replace it with more perfect methods, such as the spontaneous culture and silica plate methods, which are to be used simultaneously; the former giving indication chiefly concerning the activity and the latter concerning the density of the population." Winogradsky and Ziemiecka (1928) state that the Beijerinck solution method permits the distinction only between two categories of soil: ". . . the one giving *Azotobacter* cultures, the other not. In the first case, the presence of *Azotobacter* germs in the soil was proved, but not their absence in the second, for the germs could well be present in the soil and not develop in the solution culture."

Beck (1935) made parallel determinations on nine soils with Ashby's medium, a modification of the Beijerinck solution, and the silica-gel plate method. He found "characteristic" pellicles in Ashby's medium only in three soils which also showed *Azotobacter* colonies by the silica-gel plate method. Lochhead and Thexton (1936) compared the agar plate method and the solution method on 16 samples and obtained a general agreement between the dilution and plate methods, though the absolute numbers found were higher with the former. Martin, Walker and Brown (1937) state: "Inasmuch as the *Azotobacter* are strict aerobes, they do not grow well in liquid media which provide partly anaerobic conditions, and the results of tests thus obtained certainly are not representative of those which would be obtained under the natural conditions existing in the soil. In the film which is formed upon the surface of the medium by these organisms, there is competition for the mannitol available, and, in addition, amoebae flourish in the solution and may devour the *Azotobacter* cells. Furthermore, the liquid medium frequently becomes charged with carbonic and butyric acids which may limit *Azotobacter*

development so that the brown film characteristic of these organisms is formed only when the soil tested contains a relatively large number of the specific organisms. It is possible, therefore, for a soil to contain large numbers of the organisms, and yet they may escape detection by the liquid culture test." Also: "Frequently soils which show no *Azotobacter* when tested in a liquid culture may be found well supplied with these organisms when tested by the soil plaque method or the silica-gel plate method."

Jensen (1940) carried out 102 simultaneous plate counts and solution tests from which he concluded that the solution method may be better adapted for detecting a sporadic presence of *Azotobacter* than the plate count method.

A few comparisons only appear to have been made of the soil plaque with either of the other two methods. Winogradsky (1928) suggests that a soil may be "temporarily inactive" and give no spontaneous culture on the soil plaque, whereas a plate count may reveal the presence of *Azotobacter*. Turk (1935) compared the number of colonies which developed, using the plate and soil plaque methods, and found no definite correlation in the number of colonies developing, though the plate and plaque methods in general gave comparative results in colony counts. Martin, Walker and Brown (1937) compared the soil plaque method with the agar plate method and found the two methods qualitatively in agreement in 169 determinations.

Referring to unpublished data, Peterson and Goodding (1941) state: "Most of the evidence, then, seems to show that some methods are better than others for establishing the presence of *Azotobacter* in soils . . . . ."

There are, thus, disconcerting differences of opinion with respect to the efficacy of the methods available for the detection of *Azotobacter*. In particular, it is desirable that precise information be obtained on the sensitivity of the soil plaque and Beijerinck solution methods for qualitatively indicating the presence of *Azotobacter*. In these investigations, accordingly, a simultaneous comparison of cultural techniques has been made for the qualitative determination of *Azotobacter*, while plate counts have been made simultaneously to determine the number of *Azotobacter* cells present.

RESULTS OF COMPARISON OF TECHNIQUES

A total of 136 soil samples has been examined using simultaneously the soil plaque, the Beijerinck solution and the plate count techniques. The eight possible combinations resulting from the comparison of the three techniques, together with the respective number of determinations occurring in each group, are as follows:—

S. B. P.	S. B. P.	S. B. P.	S. B. P.	S. B. P	S. B. P.	S. B. P.	S. B. P.
+ + +	+ + —	+ — +	— + +	+ — —	— + —	— — +	— — —
47	5	0	2	1	6	1	74

S = Soil plaque ; B = Beijerinck solution ; P = Plate count.

Altogether 354 simultaneous comparisons have been made of the Beijerinck solution technique and the agar plate method, with the following results:—

B. P.	B. P.	B. P.	B. P.
+ +	+ —	— +	— —
241	29	1	83

In the simultaneous comparisons of the three techniques, of 62 positive possible the Beijerinck solution detected 60 and the plate count 50. The soil plaque method detected 53 of the 62 possible. In the simultaneous comparison of the Beijerinck solution and the agar plate (plate count) methods, of 271 positive possible, the Beijerinck solution detected 270 and the plate count 220.

Technical criticisms of the solution method appear to be without foundation, and this method, devised by Beijerinck in 1895, remains an accurate and sensitive means for the detection of *Azotobacter,* with the exception, naturally, of strains incapable of using mannitol, such as that described by Smith (1935).

In the majority of Beijerinck solution determinations a typical *Azotobacter* pellicle was formed on the surface of the solution and presence of the organism was confirmed by microscopic examination. Rarely, a doubtful pellicle appeared; i.e., a non-continuous pellicle or simply an occasional grey-brown or dark-brown to black patch on the surface of the solution. The presence or absence of *Azotobacter* in these instances was determined microscopically and/or by streaking from these areas to mannitol or dextrine agar.

The Beijerinck solution test is conveniently handled and commends itself for qualitative determinations in bacteriological survey work.

The results obtained with the soil plaque do not support Winogradsky's contention that some soils may be "temporarily inactive" and indicate that *Azotobacter* may be detected as frequently by the soil plaque as by the agar plate method. Early in these investigations it became apparent that no precise correlation existed between the amount, or intensity, of surface growth and the actual number of cells per gram of soil as shown by the agar plate method. The explanation of this may lie in the direction suggested by Winogradsky—that plaques indicate "activity" of *Azotobacter* and not the "density" of cells. To make an appreciation of the surface growth on a soil plaque the following points, however, are worthy of attention. A surface colony may originate from the development on or near the surface of a single cell, or from the development on or near the surface of an aggregate of several cells (i.e., a colony), and colonies may vary in the number of individual cells. The possibility may exist, then, of two soils with similar colony numbers but with different numbers of individual cells producing a similar intensity of macroscopic growth on a soil plaque. Further, the suitability of a soil for the preparation of a plaque, and as a corollary its suitability for the production of macroscopic growth, depends

largely on its physical characteristics. A smooth surfaced plaque with the moisture held evenly is most suitable for the growth of *Azotobacter*. It is suggested, then, that a clay loam may show a greater intensity of macroscopic growth (i.e., Winogradsky's "activity") than a sand or sandy loam containing a population of *Azotobacter* of similar magnitude. Finally, the moisture content of a plaque may condition the amount or intensity of surface growth, and the optimum moisture content for the organism may be difficult to assess casually with different soils.

The results obtained with Queensland soils do not support Winogradsky's classification of "temporarily inactive" soils, and his concept of "activity" would appear open to inquiry.

Fungi occasionally developed on the surface of soil plaques in small colonies. No bacterial growth of any significance other than *Azotobacter* appeared.

Disadvantages associated with the soil plaque method are that sandy soils require special treatment with kaolin and that difficulty may be experienced with heavy textured soils in assessing correctly the amount of moisture. An excess of moisture with clays and clay loams produces anaerobic conditions, and gas production, mainly due to the activity of *Clostridium*, causes the formation of "soil blisters." Advantages are the speed of determination after incubation of the plaque, the rapid segregation of soils into negative and positive types, and the possible further segregation of the latter into those with high, moderate and low numbers.

Azotobacter was detected equally well by the use of dextrine agar or mannitol agar. Jensen's method employing the former medium requires less manipulative effort and is preferred for this reason. Both methods are particularly selective for *Azotobacter*, giving large, dark-brown pigmented colonies in 72-96 hours and producing no growth other than very small colonies of other species of bacteria. No other bacterial or fungal growth produced on either dextrine or mannitol agar was of sufficient intensity to indicate that other species of nitrogen-fixing organisms were detectable on these media.

ENVIRONMENTAL FACTORS AND THE OCCURRENCE OF AZOTOBACTER AND CLOSTRIDIUM IN QUEENSLAND SOILS.

To determine the distribution of *Azotobacter* and *Clostridium* in Queensland soils, 151 soil samples have been examined. Particular attention has been given to the more extensive soil types of south-eastern Queensland and the samples represent a wide range in general and specific soil characteristics.

Azotobacter determinations have been made by the Beijerinck solution, soil plaque and agar plate methods in the majority of samples, and *Clostridium* has been determined microscopically after incubation in glucose solution, with separate determinations of spores.

EXAMINATION OF RESULTS

Statistical

To summarise the data relating to pH, moisture and the occurrence of *Azotobacter*, the results have been grouped according to three pH ranges and four moisture ranges, as shown in Table 1.

Table 1

OCCURRENCE OF *Azotobacter* IN RELATION TO SOIL pH AND MOISTURE CONTENT.

pH	Moisture Percentage				Total for All Soils	Total for Soils >10% Moisture
	0—10	10.1—20	20.1—30	30.1—41.8		
<5.9	$\frac{0}{15} = 0\%$	$\frac{2}{20} = 10\%$	$\frac{3}{19} = 15.8\%$	$\frac{1}{4} = 25\%$	$\frac{6}{58} = 10.3\%$	$\frac{6}{43} = 14.0\%$
6.0—7.0	$\frac{4}{19} = 21\%$	$\frac{18}{22} = 81.8\%$	$\frac{10}{13} = 77.7\%$	$\frac{5}{9} = 55.5\%$	$\frac{37}{63} = 58.7\%$	$\frac{33}{44} = 75.0\%$
>7.1	$\frac{5}{5} = 100\%$	$\frac{8}{9} = 88\ 8\%$	$\frac{5}{6} = 83.3\%$	$\frac{2}{3} = 66.6\%$	$\frac{20}{23} = 87.0\%$	$\frac{15}{18} = 83.3\%$
—	$\frac{9}{39} = 23\ 1\%$	$\frac{28}{51} = 54.9\%$	$\frac{18}{38} = 47.4\%$	$\frac{8}{16} = 50.0\%$	—	—

As judged by the marginal ratios, the proportion of positive determinations shows significant variations with soil moisture and with pH. For variation of soil moisture $\chi^2 = 9.806$ (3 D.F.) and $.05 < P < .02$. The lack of homogeneity is due to the lower proportion of positive determinations in the range 0 — 10%. The proportion of positive determinations shows a marked increase with increasing of pH value $\{\chi^2 = 49.492$ (2 D.F.), $P < .001\}$. If the determinations with soil moisture at less than 10% are excluded the values are as shown in the last column of Table 1.

The variation between the pH marginal ratios is highly significant $\{\chi^2 = 41.297$ (2 D.F.) $P < .001\}$, the significance being due to the low percentage for pH values < 5.9. There was no evidence of any significant variation between the marginal ratios for different soil moisture ranges $\{\chi^2 = .510$ (2 D.F.) $.7 < P < .8\}$. Thus, if the soil moisture content is greater than 10%, the data provide no evidence of any significant variation in the percentage of positive determinations for the various soil moisture ranges; they do provide evidence of a very significant variation in the percentage of positive determinations for the various ranges of pH values, the percentage in the class " < 5.9" being much lower than that in the other two ranges.

The figures for the two highest ranges of pH values both show a lower ratio of positive determinations in the highest soil moisture class, but the value of χ^2 calculated from the 3 x 2 table was 3.264 (2 D.F.) $.10 < P < .20$, which is not significant.

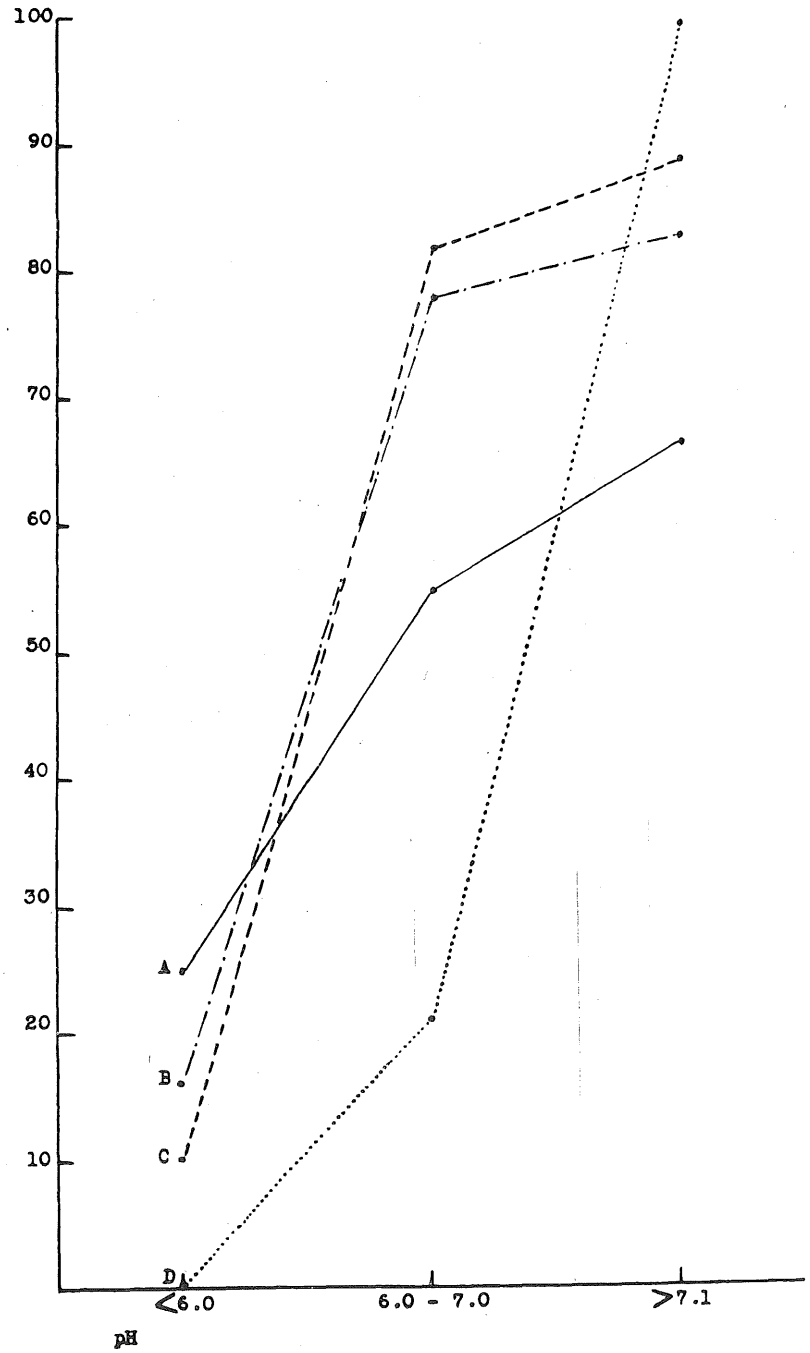

Figure 3

The occurrence of *Azotobacter* in Queensland soils in relation to moisture and pH.
A. 30.1%—40% H_2O; B. 20.1%—30% H_2O;
C. 10.1%—20% H_2O; D. 0%—10% H_2O.

The percentages of positive determinations in relation to pH and moisture are presented graphically in Figure 3.

The Influence of H Ion Concentration on the Occurrence of Azotobacter.

Investigational work on the influence of hydrogen ion concentration on the distribution of *Azotobacter* in soils is of some magnitude and has been reviewed by Jensen (1940). The results of many workers indicate that soil reaction considerably controls the occurrence of the organism, with a more or less critical point at pH 6.0 below which *Azotobacter* cells are present in small numbers or not at all. However, Peterson and Goodding (1941), using a modified Winogradsky soil plaque, found *Azotobacter* present in 92.6% of 316 Nebraska soils and found no indication of a relationship between pH and the presence of *Azotobacter*. (In view of these unusual results, Peterson and Goodings' modification of the Winogradsky soil plaque was employed in the present investigations.)

The number of Queensland soils examined and the number *Azotobacter*-positive are shown in Figure 4.

For comparison, the data on occurrence of *Azotobacter* in relation to pH obtained in the present investigations are tabulated in Table 2 together with similar data obtained from Victorian (Swaby, 1939) and New South Wales soils (Jensen, 1940; Jensen and Swaby, 1940).

Table 2

Azotobacter IN AUSTRALIAN SOILS IN RELATION TO pH.

pH Range	Victoria		New South Wales		Queensland	
	Total	*Azotobacter*—positive	Total	*Azotobacter*—positive	Total	*Azotobacter*—positive
4.0—5.0	9	1	5	0	18	1
5.1—6.0	35	4	69	7	44	6
6.1—7.0	18	6	47	15	59	34
7.1—8.0	15	8	19	14	22	20
8.1—9.0	3	2	3	1	3	2
TOTAL	80	21	143	37	146	63

Below pH 6.0 the results obtained in the three States are more or less in agreement. Above pH 6.0 the Victorian soils were 44.4% positive, the New South Wales 43.3% positive, and the Queensland 66.7% positive.

The Influence of Moisture on the Occurrence of Azotobacter.

Analysis of the data has been based on the presence or absence only of the organism. Due to the variation in populations from place to place in a soil (Swaby, 1939), in addition to possible seasonal effects on colony size (Rossi *et al.*, 1936), deductions based on numbers of cells in a series of single determinations made over different periods of the year would be liable to error.

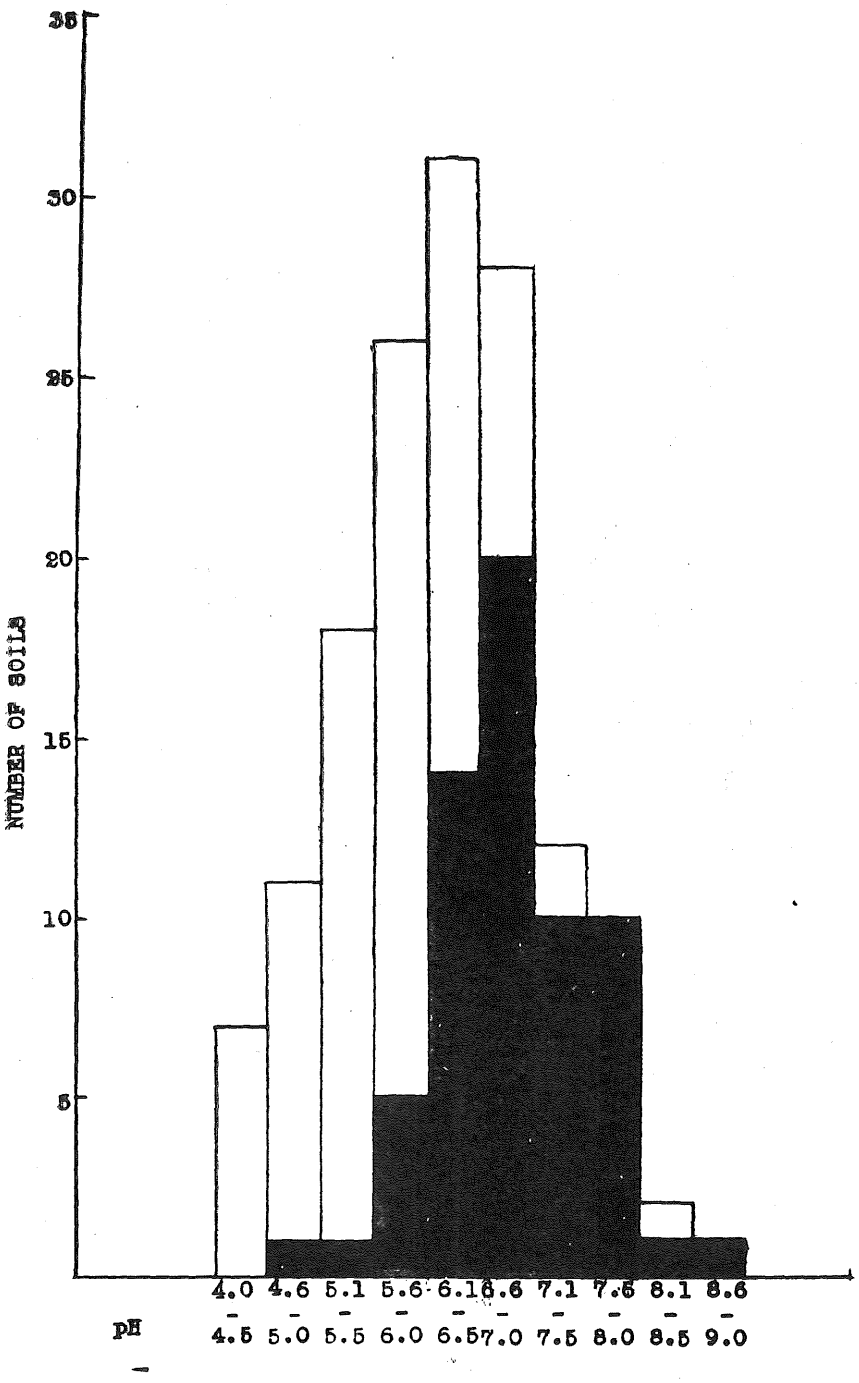

Figure 4

The number of soils examined and the number *Azotobacter*-positive in various pH ranges.

Examination of these data has shown that a lower percentage of positive determinations was obtained when the soil moisture was below 10%, but there was no evidence of any significant variation of percentage of positive determinations for various soil moisture ranges above 10%.

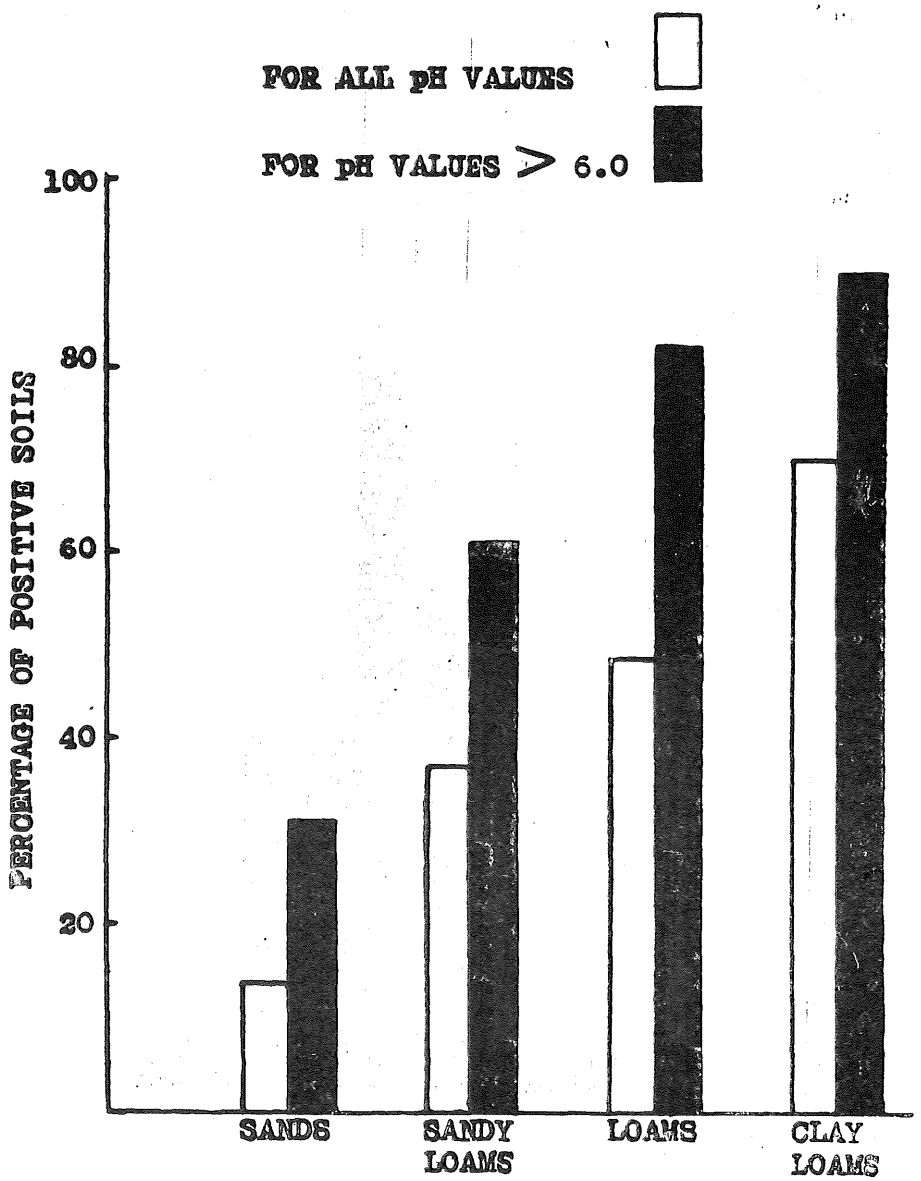

Figure 5
The occurrence of *Azotobacter* in relation to soil type.

Soil Type and the Occurrence of Azotobacter.

In the Queensland soils examined, an effect of soil type on the occurrence of *Azotobacter* has been revealed, with an increasing percentage of positive samples being recorded in the order: sands, sandy loams, loams, clay loams. The percentage of positive samples for all pH values and for pH values greater than 6.0 are shown in Figure 5. Only two clays were examined and both contained *Azotobacter*.

Swaby (1939) found in Victorian soils: "There was no conclusive evidence that the poorly-aerated soils such as clay and clay loam were more deficient in *Azotobacter* or richer in *Cl. butyricum* than the better-aerated types like sand, sandy loam and loam."

In the present investigations there was a definite trend towards a larger percentage of positive samples as the content of soil colloids increased—i.e. as the texture became heavier.

The black earths of the Darling Downs, Fassifern Valley, Biloela and Gatton districts are rich in *Azotobacter,* while the organism is either absent from or present in very low numbers in the granitic sands of the Stanthorpe district, the weakly podsolized coastal soils and the red earths of the Kingaroy and Yarraman areas. Influencing the presence of *Azotobacter* in the black earths are the favourable pH and, probably, the relatively high phosphate and organic matter content of these soils.

The distribution of *Azotobacter* in soils of south-eastern Queensland is shown in Figure 6.

Jensen (1940) found no direct correlation between the general productivity of the soils examined and the presence or absence of *Azotobacter*. He found *Azotobacter* absent or only sporadically present in the Wimmera soils of Victoria, which are representative of the most fertile type of wheat soils in Australia. If fertility may be defined as the capacity of a soil to tolerate continuous cropping over a large number of years without the necessity for adding artificial fertilizers, then the black earths may be accepted as the most fertile of the soil types examined in the Queensland investigations, and *Azotobacter* was found more frequently in these soils than in others.

The greater proportion of Queensland's wheat crop is taken from the black earth soils, and Jensen's statement that "*Azotobacter* is relatively sparsely represented in Australian wheat soils" would require modification to include Queensland wheat soils.

No determinations of organic matter have been made, but accepting colour as a general indication the clay loams and clays examined contained relatively higher percentages of organic matter than the sands and sandy loams.

Distribution of Clostridium in Queensland Soils.

One hundred and forty-three samples have been examined for the presence of *Clostridium*. In three soils only, a medium sand and two coarse

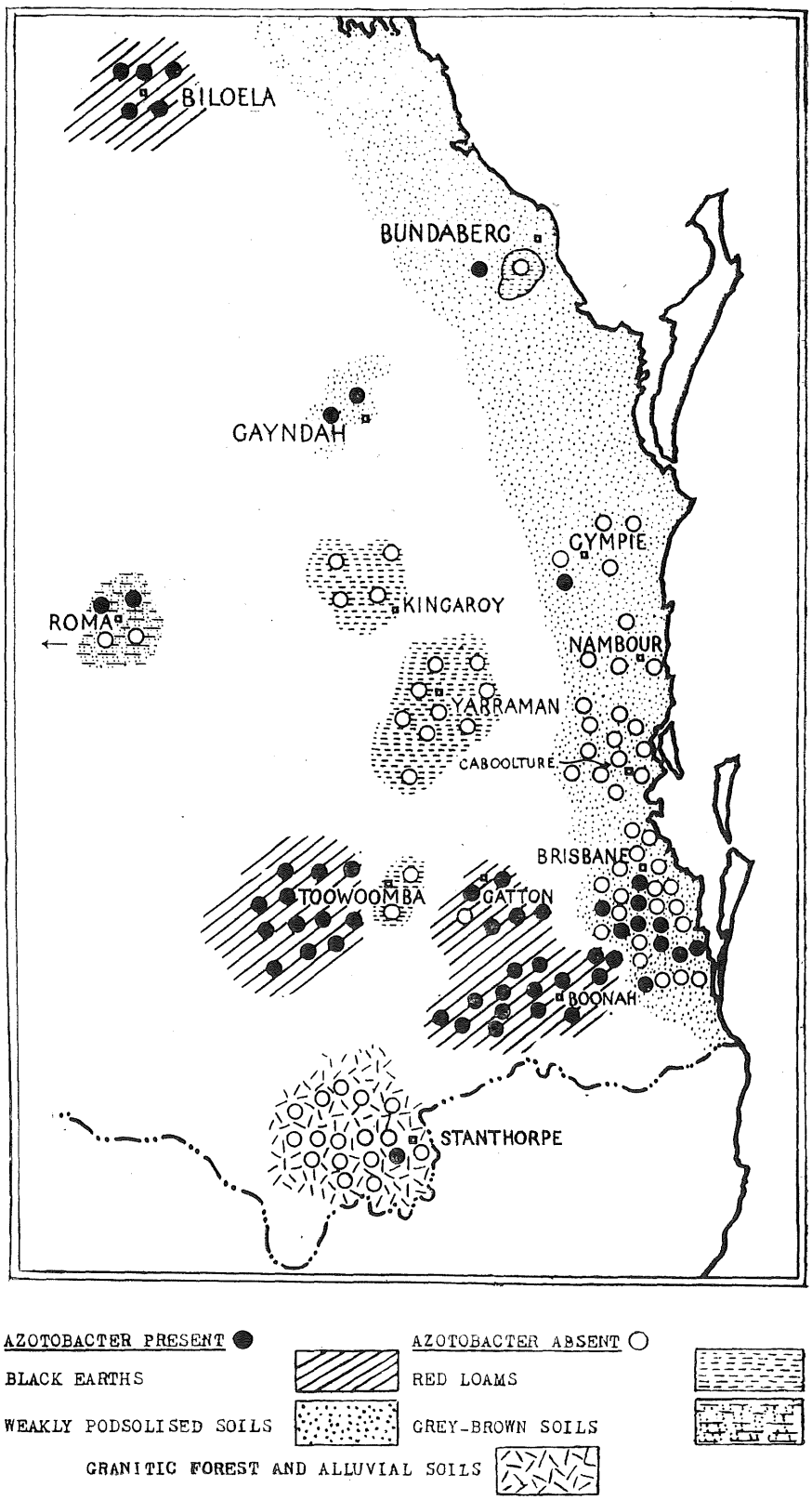

Figure 6
The distribution of *Azotobacter* in soils of south-eastern Queensland.

sands from the Beerwah district, was *Clostridium* not detected. In five other soils the presence of *Clostridium* was not readily detected, the organisms being absent either in the determination of spores or of spores plus vegetative cells. Three of these samples were sands and three sandy loams; five of the six samples were obtained from either the Beerwah or the Stanthorpe districts. Spore forms of *Clostridium* were demonstrated in practically all samples.

The results show that species of *Clostridium* are commonly present in Queensland soils. Precise conclusions cannot be drawn in the absence of quantitative determinations, but it would appear that while the presence, qualitatively, of *Clostridium* is unconnected with any specific environmental factor or factors, the organisms may occasionally be absent from the sands of the wallum areas and from the granitic sands of the Stanthorpe district.

Occurrence of Azotobacter and Clostridium in Virgin Soils.

The question of the occurrence and importance of free-living nitrogen-fixing bacteria in the nitrogen economy of virgin soils is of considerable interest. Jensen (1940) has suggested that "the importance of non-symbiotic nitrogen fixation in nature is probably largely confined to uncultivated soils where no crops are carried away and the vegetable debris is allowed to decompose in situ."

Forty-eight of the determinations of *Azotobacter* were made on uncultivated soils. Of these 10, or 20.8%, contained the organism. Summarized data on the occurrence of *Azotobacter* in virgin soils are shown in Table 3, where, for comparative purposes, similar data from cultivated soils are included.

Solely from the point of view of the occurrence it would appear that the role of *Azotobacter* is not more impressive in virgin soils than in cultivated soils in Queensland.

Table 3

OCCURRENCE OF *Azotobacter* IN VIRGIN AND CULTIVATED QUEENSLAND SOILS.

pH	Virgin Soils			Cultivated Soils		
	Total Examined	Number *Azotobacter* present	Percentage *Azotobacter* present	Total Examined	Number *Azotobacter* present	Percentage *Azotobacter* present
<6.0	27	1	3.7	35	6	17.1
>6.0	21	9	42.8	63	47	74.6

Two of the soils which gave a negative test for *Clostridium* were virgin soils from the wallum area, and two of the soils in which *Clostridium* was not readily detected were also wallum soils. *Clostridium* was present in all other virgin soils examined.

Occurrence of Azotobacter and Clostridium relative to Rhizobium.

Three soil samples, from wallum soils, were taken deliberately from the rhizospheres respectively of *Mirbelia reticulata*, *Gompholobium virgatum* and *Pultenaea villosa*. These species are well nodulated and it has been shown (McKnight, 1945) that the isolates of *Rhizobium* from *M. reticulata* and *P. villosa* are capable of fixing nitrogen in association with *Vigna unguiculata*. A generalised conclusion in respect of the relative roles of the three nitrogen-fixing organisms in wallum soils may be possible. *Azotobacter* is absent, *Clostridium* may be absent, and strains of *Rhizobium* capable of fixing nitrogen are present.

In the virgin granitic sands of the Stanthorpe area *Azotobacter* is not a component of the soil flora, *Clostridium* is generally present, and native strains of *Rhizobium*, demonstrated capable of fixing nitrogen, commonly occur in indigenous legumes.

There is little doubt that *Rhizobium* in these areas plays a more significant role in the nitrogen economy of the soil than do the two free-living organisms.

General

The only species of *Azotobacter* encountered in the Queensland soils examined was *Az. chroococcum* Beijerinck, forming characteristic brown to dark-brown colonies on dextrine or mannitol agar. Lipman's *Az. beijerinckii*, now considered identical with *Az. chroococcum* (Bergey, 1948), was present in three soils only—a dark grey-brown fine sand to sandy loam in the University grounds, Brisbane, in a grey-brown sandy loam in an experimental plot in the Domain, Brisbane, and in a dark grey clay loam to light clay from Kuraby. Colonies of this strain formed a sulphur yellow pigment on dextrine or mannitol agar.

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